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## Proteinuria-associated renal injury and the effects of intervention in the renin-angiotensin-aldosterone system

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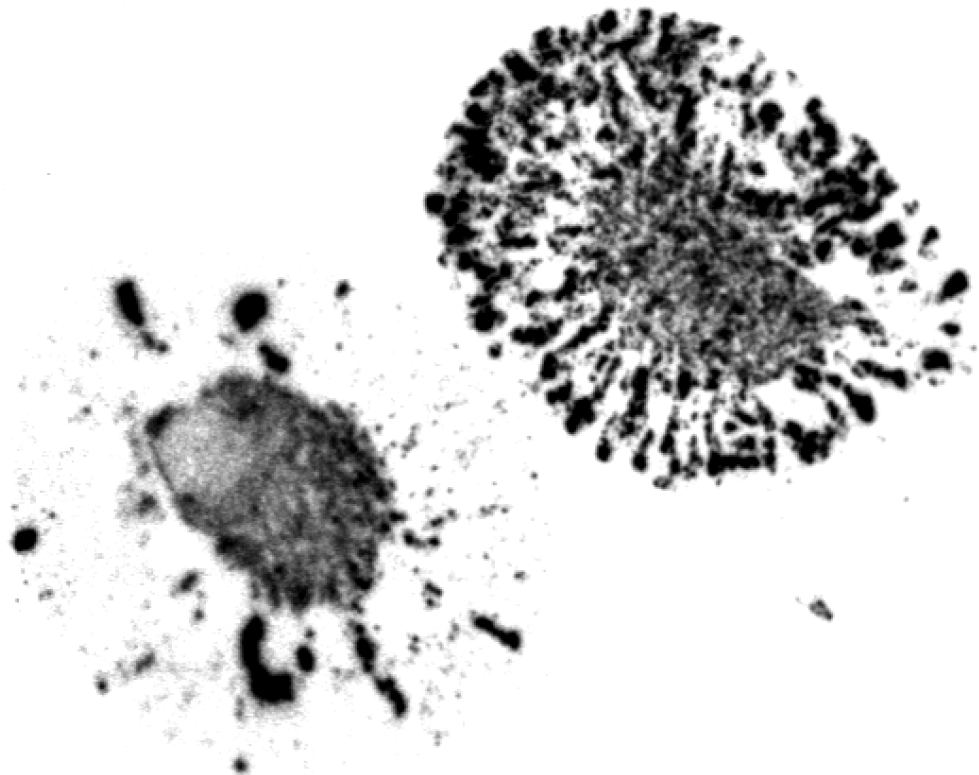
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## Chapter 5

# **Induction of glomerular heparanase expression in rats with adriamycin nephropathy is regulated by reactive oxygen species and the renin-angiotensin system**

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## **ABSTRACT**

Heparan sulfate (HS) plays an important role in the regulation of glomerular permeability. Loss of these anionic molecules in the glomerular basement membrane (GBM) can lead to proteinuria and finally to end stage renal disease. Heparanase has been implied in loss of HS in several diabetic and non-diabetic proteinuric conditions. In this study, we analysed the role of heparanase in the degradation of HS in the GBM in adriamycin nephropathy (AN), a model of chronic proteinuria-induced renal damage.

Expression of HS, heparanase and the core protein agrin to which HS is attached, was determined on frozen kidney sections from rats with AN in three different experiments. First, expression was examined in a time course study at 6 week intervals until week 30 in a unilateral model of AN, in relation to the development of proteinuria and renal damage. Second, rats were treated with the hydroxyl radical scavenger dimethylthiourea (DMTU) during disease induction by adriamycin, and its effect on the development of proteinuria, HS expression and heparanase was assessed after 4 weeks. Finally, in a third experiment AN was induced and after 6 weeks a renal biopsy was taken. Subsequently, rats were treated with angiotensin II receptor 1 antagonist (AT1A) or vehicle for two weeks and then sacrificed and their kidneys investigated. Heparanase expression was increased in the glomeruli of rats with AN, which was correlated with a reduction of HS at all time points in all experiments. The upregulation of heparanase was already observed after 6 weeks. Treatment with the radical scavenger DMTU during disease induction prevented the increased heparanase expression and the decrease of HS in the GBM and prevented albuminuria. Finally, treatment of established proteinuria with AT1A significantly reduced heparanase expression and restored HS expression in the GBM.

In conclusion, this study shows an association between increased heparanase expression and reduction of HS in the GBM by several approaches in the AN model. The effects of DMTU suggest a role for reactive oxygen species (ROS) in the increased heparanase expression and decreased HS expression. Interestingly, antiproteinuric treatment in established disease by AT1A decreased heparanase expression and restored HS expression, which may contribute to the renoprotective effects of this regimen. These studies suggest the involvement of ROS and angiotensin II in the modulation of GBM permeability through heparanase and HS.

## INTRODUCTION

The glomerular basement membrane (GBM) consists of many extracellular matrix proteins, including heparan sulfate proteoglycans (HSPGs). Loss of negatively charged heparan sulfate (HS) molecules, which are covalently attached to the core proteins of either agrin, perlecan or collagen XVIII, results in altered charge-dependent permeability of the GBM [1]. The importance of HS in the charge-dependent permeability of the GBM has been demonstrated in several studies. First, digestion of HS by heparitinase resulted in increased permeability of the GBM for ferritin and albumin [2;3]. Second, injection of a specific monoclonal antibody against HS caused a massive albuminuria in rats [4]. Third, loss of anionic HS has been reported in several human and experimental glomerulopathies, which was inversely correlated with the degree of proteinuria [5;6]. It is suggested that proteinuria-induced renal damage is associated with loss of glomerular HS in diabetic and non-diabetic renal disease [7-9].

HS loss in proteinuric renal disease can be attributed to several factors (reviewed in [1]) such as depolymerization of HS by reactive oxygen species (ROS), masking of HS by nucleosome/Ig complexes, reduction of both HS production and sulfation due to hyperglycemia, complement mediated cleavage of HS and proteolytic cleavage of the HS attachment site on the core protein by enzymes.

Heparanase is an endo- $\beta$ (1,4)-D-glucuronidase involved in the cleavage of HS and hence is associated with extracellular matrix degradation and tissue remodelling [10-13]. A recent study in patients with diabetic nephropathy suggests that loss of HS in the GBM is attributable to accelerated HS degradation by increased heparanase expression [14]. Studies in experimental renal diseases, i.e. passive Heymann nephritis, puromycin aminonucleoside nephrosis and anti-GBM nephritis suggest that heparanase may be involved also in non-diabetic proteinuric disease [15-18]).

In chronic proteinuric renal diseases blockade of the renin-angiotensin system (RAS) reduces proteinuria and thereby reduces progressive renal function loss. In adriamycin nephropathy (AN), a model of chronic proteinuric renal damage, we previously reported a decrease of HS expression, which was partially caused by hydroxyl radicals [7]. Furthermore, we found that RAS-blockade protects against loss of HS in established adriamycin nephropathy [9]. However, whether changes in heparanase may be involved in the decrease of HS expression in AN, and/or the effects of RAS-blockade is unknown.

In the present paper therefore we first determined the time course of heparanase and HS expression in relation to the development of proteinuria and renal structural damage in AN. For this purpose we used the unilateral variant of the AN model in order to allow a good resolution over time. Next, the effect of scavenging of ROS during the period of induction of AN on heparanase and HS expression, and on proteinuria was studied. Finally, we evaluated the potential of antiproteinuric treatment with an angiotensin II type 1 receptor antagonist (AT1A) to restore the disbalance between heparanase and HS in established AN with persistent proteinuria.

## METHODS

### *Animals and experimental design*

Male Wistar rats were housed in a temperature-controlled room with a 12 hour light-dark cycle and with free access to food and water. 24-hour urine was collected 2-weekly in metabolic cages, with measurement of water and food intake. Surgical procedures took place under isoflurane anesthesia in N<sub>2</sub>O/oxygen (1:2). Systolic blood pressure (SBP) was measured weekly by the tail cuff method in conscious rats [19]. At the end of the study the abdominal aorta was cannulated, a 2 ml blood sample was taken and kidneys were perfused *in situ* with saline and removed. Proteinuria was measured on a BNII third generation nephelometer (Dade Behring, Mannheim, Germany) by using a 20% tri-chloroacetic acid (TCA) solution. The Committees for Animal Experiments of the University of Groningen and the Radboud University Nijmegen, The Netherlands approved all studies.

*Experiment 1:* Unilateral AN was induced by temporarily clipping the left renal artery and vein through a midline abdominal incision [19], followed by adriamycin (1.5 mg/kg body weight (BW)) injection via the tail vein. After 12 minutes, when adriamycin has been cleared from the circulation [20], the clamp was removed. To study expression of heparanase and HS, and renal damage over time, 8 rats (at each time point) were sacrificed at weeks 6, 12, 18, 24 and 30. In this experiment the right kidney - exposed to adriamycin - was compared to the control, i.e. non-exposed, left kidney. Previously, we showed that the clipped left kidney did not differ from healthy control kidneys, even not after 30 weeks [21].

*Experiment 2:* Bilateral AN was induced by intravenous injection of 5 mg/kg BW adriamycin via the tail vein. One group of animals (n=8) received an initial intraperitoneal injection of dimethylthiourea (DMTU; 500 mg/kg BW; Sigma) 6 hours before adriamycin injection, followed by treatment with DMTU (1.25 mg/kg BW) twice a day for 7 days. The control group (n=7) received saline instead of DMTU. Rats were sacrificed 4 weeks after the injection of adriamycin. Urine was collected at week 0 and 4 for determination of urinary albumin excretion.

*Experiment 3:* Bilateral AN was induced by intravenous injection of adriamycin (2 mg/kg BW) via the tail vein. At week 6 a renal biopsy was performed via a dorsolateral incision. After removal of a part of the lower pole from the left kidney, gelfoam (Spongostan®, Ferrosan, Copenhagen, Denmark) was applied for haemostasis. After recovery, rats were treated with the angiotensin II type 1 receptor antagonist L158,809 (AT1A, 150 mg/l drinking water, n=20) or vehicle (VEH, n=10). In previous experiments the biopsy did not affect the course of renal damage [9;22]. Treatment was continued until sacrifice at week 12. Eight healthy rats that were sacrificed at week 6 were used as time controls.

**Immunofluorescence staining**

To determine heparanase, HS and agrin core protein expression, indirect immunofluorescence (IF) staining was performed on 2µm cryostat kidney sections. Tissue sections were fixed in 100% acetone for 10 minutes at 4°C, followed by 1 hour incubation of primary antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide at room temperature. After washing in PBS, the appropriate secondary antibodies were diluted in PBS and incubated for 1 hour at room temperature, with 5% normal rat serum and 10% normal goat serum. Specific primary and secondary antibodies that were used are summarized in Table 1. Subsequently, the sections were washed in PBS and embedded in Vectashield mounting medium H-1000 (Vector Laboratories Inc., Burlingame, CA).

To investigate which glomerular cell type was responsible for the expression of heparanase in AN, sections were double stained with anti-heparanase and: 1) anti-agrin, to distinguish between the outside of the GBM covered by podocytes or the inside with endothelial cells lining the GBM, 2) anti-synaptopodin, which stains the cytoskeleton of podocytes, and 3) anti-Thy 1.1, which is a mesangial cell marker. To investigate in which the tubular cell type heparanase is expressed, double stainings were performed with anti-heparanase and 1) anti-aquaporin 2, a marker for collecting ducts; 2) anti-calbindin D-28k, a marker for distal convoluting tubules and 3) anti-P-glycoprotein, a marker for proximal tubules. Confocal laser scanning microscopy (CLSM; Leica, Heidelberg, Germany) was used to evaluate the stainings.

**Table 1. Antibodies used for immunohistochemistry**

Antibody	Specificity and epitope	Dilution	Ref/Company	Secondary antibody <sup>#</sup>
HPA1	Rabbit anti- heparanase: 50kDa/8kDa heterodimer	1:100	ProsPec-Tany Technogene LTD, Israel	Goat α-rabbit IgG Alexa 594
JM403	Mouse anti-rat HS: N-unsub- stituted glucosamine unit	1:300	[4;23]	Goat α-mouse IgM Alexa 488
MI91	Hamster anti-agrin core protein: N-terminus	1:800	[24]	Goat α-hamster IgG Cy3
OX7	Mouse anti-Thy 1.1: mesangium cells	1:300	PharMingen, USA	Goat α-mouse IgG Alexa 488
G1D4	Mouse anti-synaptopodin: podocyte cytoskeleton	1:1	Progen Biotechnik, Germany	Goat α-mouse IgG Alexa 488
300	Mouse anti-calbindin D-28k: distal convoluted tubules	1:300	Swant, Switzerland	Goat α-mouse IgG Alexa 488
AQP2	Guinea pig anti-aquaporin 2: collecting duct	1:300	Kindly provided by Dr. P. Deen [25]	Goat α-guinea pig IgG Alexa 488
C219	Mouse anti-P glycoprotein: proximal tubules	1:20	Abcam, UK	Goat α -mouse IgG Alexa 488

<sup>#</sup>Secondary Alexa antibodies were all purchased from Invitrogen, (Molecular Probes, Breda, The Netherlands) and were used in a dilution of 1:200; Cy3 labeled antibody was obtained from Jackson ImmunoResearch Laboratories (Cambridgeshire, UK), dilution 1:800.

***Quantification of immunofluorescence and histochemistry***

The kidney sections were randomly coded and evaluated by two independent observers on a Zeiss Axioskop microscope (equipped with an epi-illumination). For agrin and HS expression, linearity of the GBM was scored at an arbitrary scale of 0-10 (0= no staining, 1= 10% linear GBM staining, etc, with a maximum score of 10 for 100% staining). Heparanase expression was scored from 0-5 arbitrary units (A.U.) for staining intensity.

Focal glomerulosclerosis (FGS) was scored semi-quantitatively on periodic acid-Schiff (PAS) stained paraffin sections (4  $\mu$ m), as described in [26]. FGS lesions were defined as glomerular areas with mesangial expansion and adhesion formation simultaneously present in one segment. Scoring was performed on a scale of 0 to 4 in 50 glomeruli per kidney moving from outer to inner cortex.

***Statistical analysis***

Data are expressed as median and 95% confidence intervals. Differences between groups were determined by Kruskal-Wallis and Mann-Whitney U test. Linear regression was performed to detect whether proteinuria and heparanase and HS expression were associated. Analyses were performed using SPSS version 12.0 and GraphPad Prism, version 4.0 software. Statistical significance was regarded when  $p < 0.05$ .

**RESULTS*****Clinical and morphological data***

Data on proteinuria and blood pressure for the time-course study (Experiment 1) and the intervention study (Experiment 3) are shown in Table 2 and 3, respectively. In unilateral AN proteinuria was increased at all time points and blood pressure was normal. The score for focal glomerulosclerosis (FGS) was increased in the adriamycin-exposed kidney compared to the non-exposed control kidney and progresses over time. When rats were treated with DMTU before and 1 week after induction of bilateral AN, albuminuria at week 4 was lower compared to saline treated AN animals (255 (185-276) mg/24h vs 353 (274-436) mg/24h),  $p < 0.05$ ). In the intervention study at week 6, i.e. before treatment with AT1A started, proteinuria was markedly increased compared to healthy controls. Treatment during 2 weeks with AT1A reduced proteinuria and blood pressure. The score for FGS did not change during the 2 weeks of treatment. In the vehicle-treated group, proteinuria and blood pressure and FGS score remained stable.

***Heparanase overexpression is associated with reduced HS expression in AN: time course***

Glomerular HS and HS expression in the exposed and non-exposed kidney, assessed by indirect immunofluorescence staining on cryostat sections, is given in Fig. 1. HS showed a nice linear staining along the GBM in control kidneys (Fig. 1A). However, in adriamycin-exposed

kidneys, the intensity of HS expression in the GBM was reduced, whereas agrin expression remained unaltered (Fig. 1B). Semi-quantitative analysis revealed that HS was significantly reduced in adriamycin-exposed kidneys at all time points without changes over time, whereas agrin core protein expression did not differ between control and adriamycin-exposed kidneys (Fig. 3A and 3B). Heparanase expression was markedly increased in all adriamycin-exposed kidneys (Fig. 1 D) as compared to the control kidneys (Fig. 1C), which was confirmed by semi-quantitative analysis. Heparanase expression was increased significantly at all time points (Fig. 3C) without changes over time. The reduction in glomerular HS expression significantly correlated with an increase in heparanase expression ( $R^2=0.34$ ,  $p<0.001$ , Fig 3D). Taken together these results indicate that increased heparanase expression and loss of glomerular HS are early events in the time course of the adriamycin-induced nephrotic syndrome.

**Table 2.** Characteristics of the time course study in unilateral AN (Experiment 1)

	week 6	week 12	week 18	week 24	week 30
<b>UProt</b> (mg/24h)	122 (72-367)	191 (142-414)	237 (183-395)	221 (70-351)	265 (159-459)
<b>SBP</b> (mmHg)	140 (112-146)	140 (122-166)	140 (122-156)	163 (120-183)	152 (138-190)
<b>FGS control</b>	0 (0-16)	2 (0-12)	8 (2-24)	19 (2-54)	24 (2-50)
<b>FGS ADR</b>	3 (0-16)*	20 (0-48)*	43 (10-78)*	45 (6-96)*	67 (38-148)*

\*  $p<0.05$  vs controls (non-exposed kidneys); Uprot: proteinuria; SBP: systolic blood pressure; FGS: focal glomerulosclerosis in arbitrary units 0-400; ADR: adriamycin-exposed kidney

**Table 3.** Characteristics of the intervention study in bilateral AN (Experiment 3)

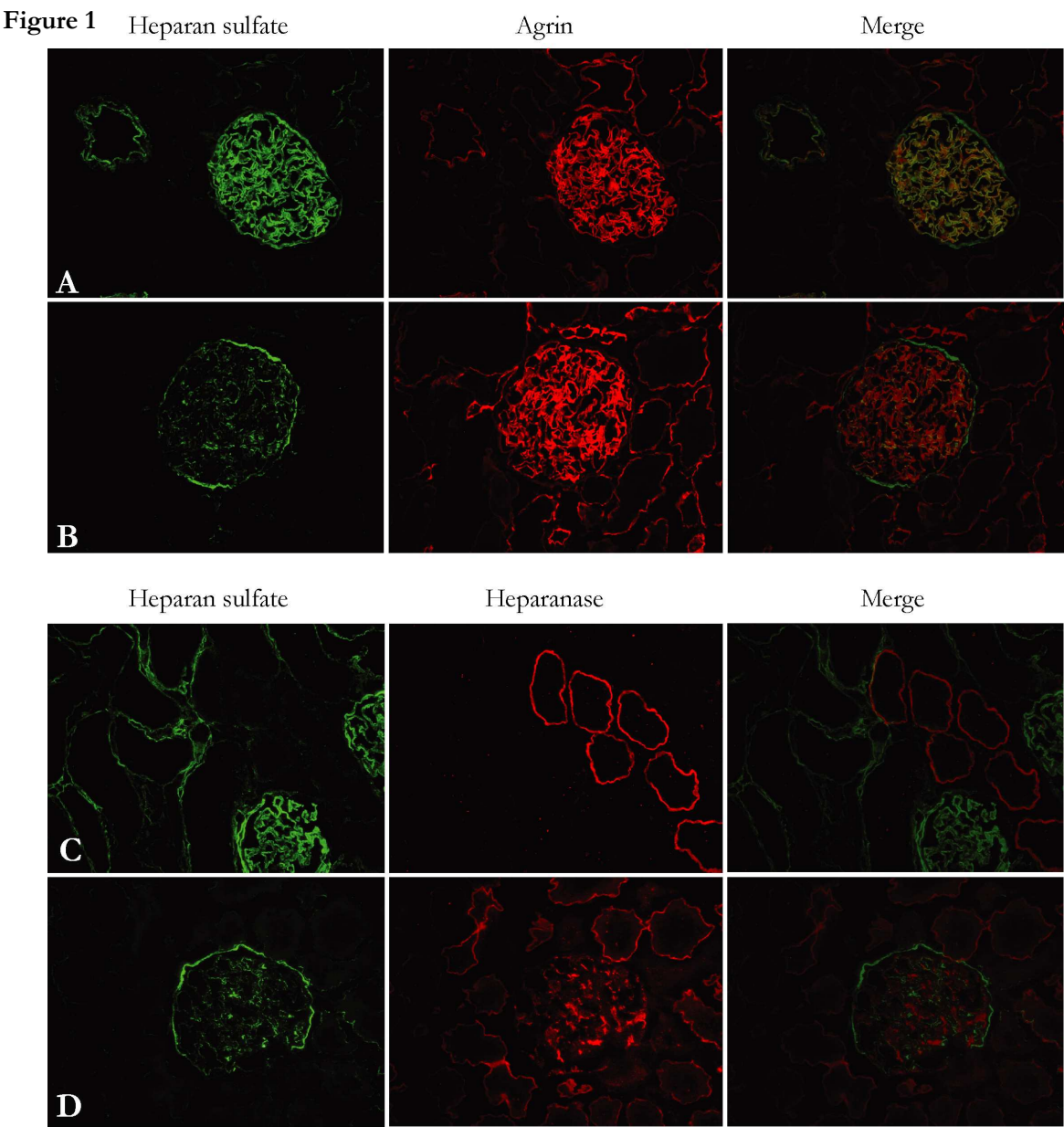
	Vehicle		AT1 antagonist		Controls
	week 6	week 8	week 6	week 8	week 6
<b>Uprot</b> (mg/24h)	777 (254-883)*	697 (257-834)	680 (250- 904)*	245 (33-638) <sup>#&amp;</sup>	13(11-17)
<b>SBP</b> (mmHg)	149 (140-171)	144 (97-156)	143 (124-164)	113 (60-148) <sup>#&amp;</sup>	140 (131-161)
<b>FGS score</b>	20 (0-30)*	30 (2-52)	12 (0-97)*	16 (2-87)	0 (0-2)

\*  $p<0.05$  vs controls, #  $p<0.05$  vs week 6, &  $p<0.05$  vs vehicle; Uprot: proteinuria; SBP: systolic blood pressure; FGS: focal glomerulosclerosis in arbitrary units (0-400)

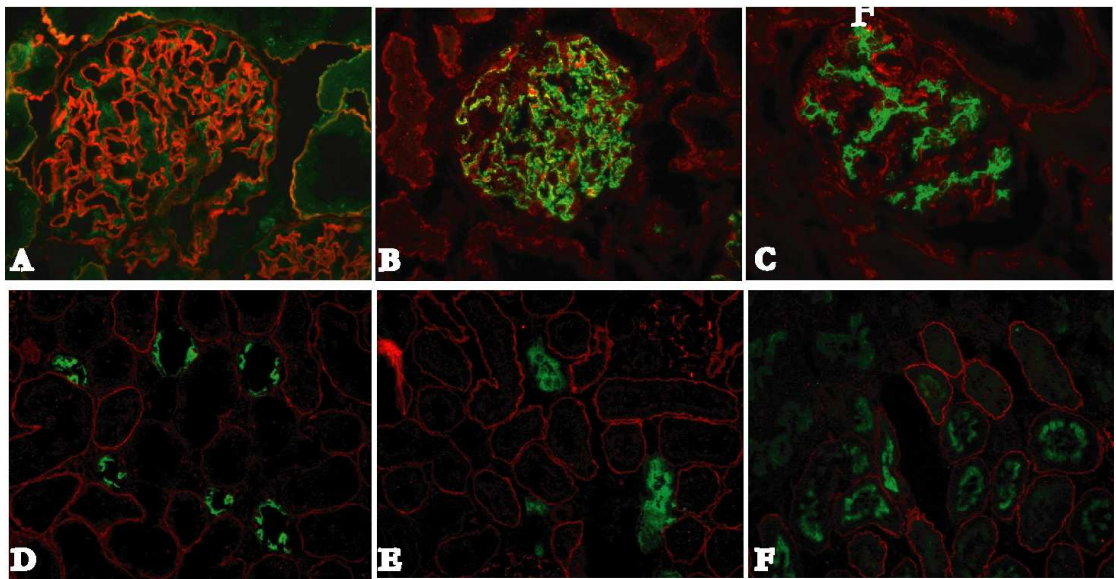
### ***Heparanase expression in glomeruli and tubules***

Heparanase expression in AN is located at the outside of the GBM, and is minimally expressed within the capillary loops (Fig. 2 A-C). Co-localization with synaptopodin confirms that heparanase is expressed by the podocytes and not by mesangium cells, since no colocalization with anti-Thy 1.1 could be observed. Heparanase is expressed in tubuli of both normal and diseased animals. Tubular HS and heparanase expression were not altered by either adriamycin injection or AT1A treatment compared to controls. Using specific tubular markers, we observed that heparanase is mainly expressed in proximal tubules. No expression was found in the distal convoluted tubules or collecting ducts (Fig. 2 D-F).





**Figure 2**



← **Figure 1.** HS(PG) and heparanase expression in AN and control kidneys (indirect immunofluorescence staining). A) JM403 staining for HS and MI91 staining for agrin core protein in a control kidney; a nice linear staining of the GBM is observed for both HS and agrin. B) HS and agrin staining in an adriamycin-exposed kidney: glomerular HS staining is decreased, whereas agrin staining remains linear. C) HS and heparanase staining in a control kidney; heparanase is present in the tubules, but absent in the glomerulus. D) HS and heparanase in an adriamycin-exposed kidney: a glomerulus with reduced HS expression and increased heparanase expression. Magnification 400x.

### ***DMTU reduces the early effect of adriamycin on HS and heparanase expression***

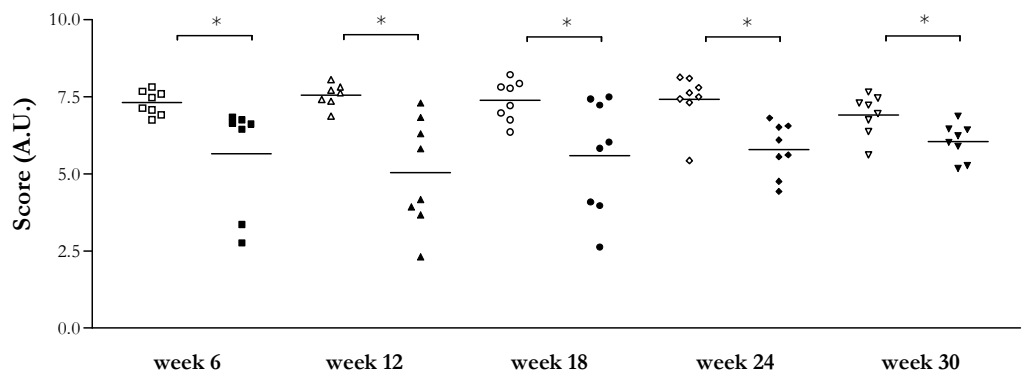
Since HS reduction in AN has been attributed to a depolymerization of HS by ROS [7], we studied whether the increased heparanase expression could be attributed to ROS, by treating rats with the hydroxyl-scavenger DMTU during disease induction by adriamycin, and the first week afterwards. At sacrifice, 3 weeks later, a loss of HS was observed in the saline-treated animals, that was partly prevented by the treatment with DMTU (Fig. 4A), whereas expression of agrin was comparable in both groups (data not shown), which is in line with prior experiments [7]. Interestingly, the glomerular heparanase expression in AN animals treated with the ROS scavenger DMTU was significantly lower compared to saline treated AN animals (Fig. 4B).

### ***Reduction of heparanase and increase in HS expression after treatment with AT1A***

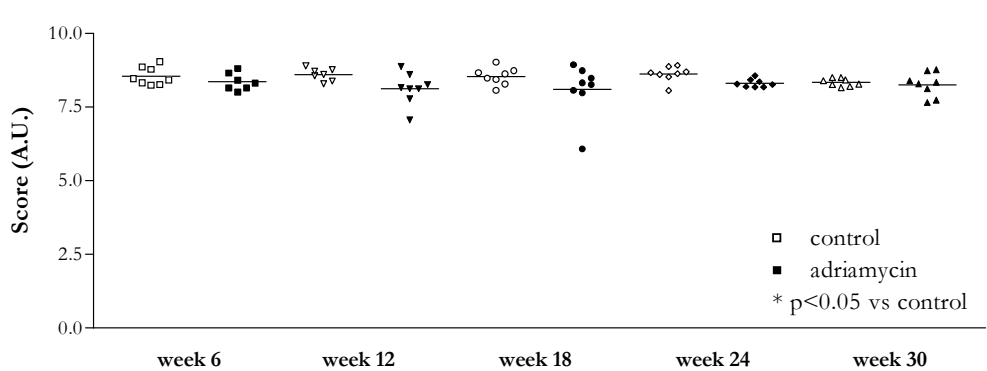
Next, we investigated whether, in established proteinuria, antiproteinuric treatment by AT1A can reduce glomerular heparanase expression along with restoration of HS expression in the GBM. HS(PG) and heparanase expression were determined in bilateral AN, before (week 6) and after 2 weeks of treatment with AT1A or VEH (week 8). Glomerular HS expression was decreased in all adriamycin-exposed animals at week 6 and heparanase expression was markedly increased, which was comparable with the results observed in the unilateral model of AN (Fig. 5). Treatment with AT1A significantly increased glomerular HS expression compared to the VEH-treated animals (Fig. 5A), which was, interestingly, accompanied by a significant reduction in heparanase (Fig. 5B). However, AT1 receptor blockade did not completely restore HS expression to normal levels as observed in the healthy controls.

← **Figure 2.** Glomerular and tubular heparanase expression in AN. For glomerular expression we used immunofluorescence double staining with anti-heparanase (in red) and (A) anti-agrin, (B) anti-synaptopodin, or (C) anti-Thy 1.1 (all in green). Glomerular heparanase is mainly expressed at the outside of the GBM, confirmed by co-localization with the podocyte marker synaptopodin. Minimal staining was observed in the capillary loops (according to the agrin/heparanase staining). No co-localization with mesangium cells was shown. For tubular heparanase expression we used immunofluorescence double staining with anti-heparanase (in red) and (D) anti-calbindin D-28k, (E) anti-aquaporin 2 and (F) anti-P glycoprotein (all in green). Tubular heparanase is mainly expressed in the proximal tubules, since it is co-localized with P-glycoprotein. No co-localization with the distal convoluting tubules and collecting ducts was shown. Magnification: 630x.

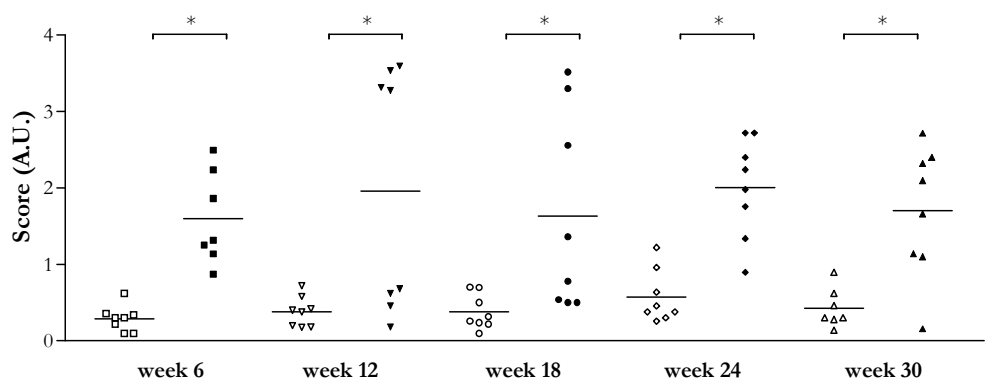
A Heparan sulfate



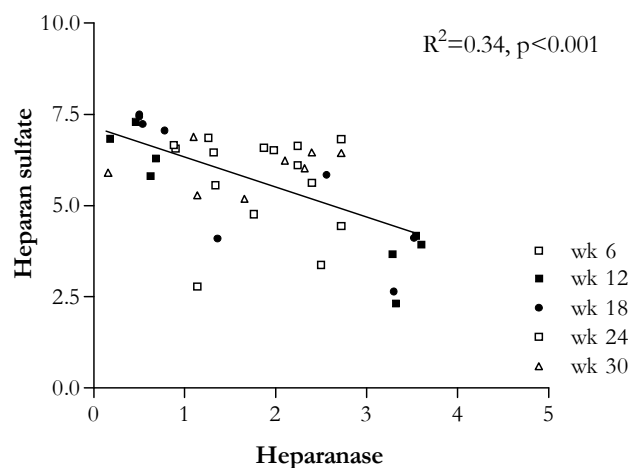
B Agrin



C Heparanase

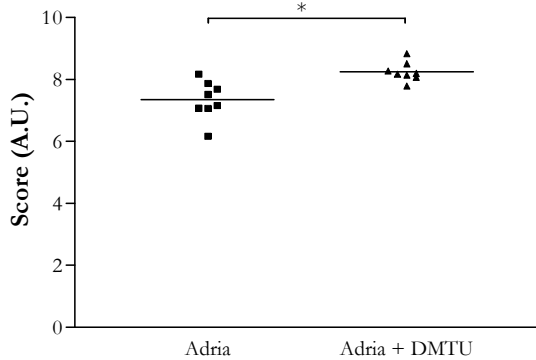


D Heparan sulfate vs heparanase

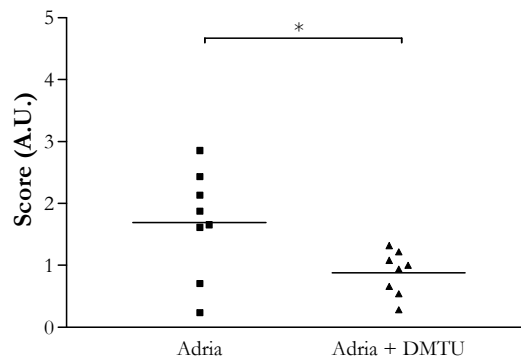


**Figure 3** Semi-quantitative analysis of glomerular HS, agrin and heparanase expression in arbitrary units (A.U.) at different time points in unilateral AN (experiment 1). A) HS expression in the GBM was significantly decreased in AN at all time points. B) Agrin core protein expression did not differ between adriamycin-exposed and control kidneys. C) Glomerular heparanase expression was up-regulated in AN at all time points. D) Correlation between HS staining and heparanase expression in unilateral AN.

### A Heparan sulfate

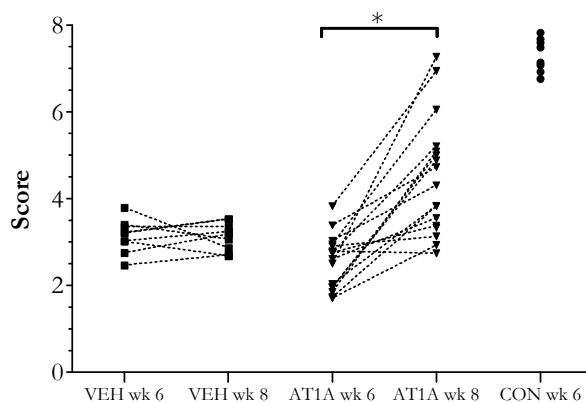


### B Heparanase

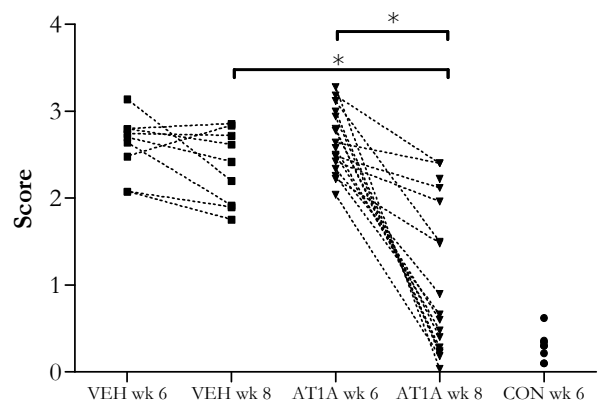


**Figure 4.** Effect of treatment with the hydroxyl scavenger DMTU before disease induction by adriamycin, and the first week afterwards (Experiment 2). A) Decrease of HS expression in AN could be partly prevented by treatment with DMTU. B) Heparanase expression was significantly lower after treatment with DMTU. \*  $p < 0.05$

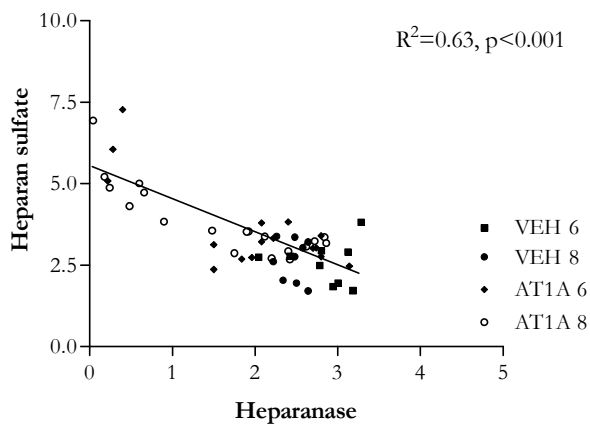
### A Heparan sulfate



### B Heparanase



### C Heparan sulfate vs heparanase



**Figure 5.** Effects of treatment with AT1A or VEH on heparanase and HS(PG) expression in bilateral AN (Experiment 3) compared to healthy control rats at week 6. Indirect immunofluorescence double staining was performed on kidney biopsies before (week 6) and after treatment (week 8). A) Glomerular HS expression was reduced before treatment, but restored after 2 weeks of treatment with AT1A. Treatment with VEH did not restore HS expression. B) After 2 weeks treatment with AT1A, heparanase expression decreased and it did not change with VEH treatment. C) Correlation between HS staining and heparanase expression in bilateral AN. \*  $p < 0.05$

For the individual adriamycin animals (VEH and AT1A at week 6 and 8), the reduction in HS expression in the GBM is significantly correlated with increased heparanase expression ( $R^2=0.63$ ,  $p<0.001$ , Fig. 5C).

## DISCUSSION

Our data demonstrate that glomerular heparanase expression is increased in adriamycin-induced nephropathy and associated with a decreased HS expression in the GBM. These changes occur early after disease induction and remain stable during follow-up, while renal structural damage progresses. We previously found reactive oxygen species (ROS) to induce loss of glomerular HS [7]; our current data show that treatment with the ROS scavenger DMTU reduces heparanase expression compared to saline-treated adriamycin animals, with subsequently a better preservation of glomerular HS expression and amelioration of albuminuria. Finally, glomerular heparanase expression in established AN was reversible by antiproteinuric treatment with RAS blockade, along with an increased glomerular HS expression.

In a previous study, we reported that by treatment with a ROS-scavenger, HS expression was not completely restored and albuminuria was not completely prevented in AN [7], suggesting that additional mechanisms are involved in the reduction of HS in this model. The present study demonstrates that heparanase expression is increased and correlated with the loss of HS in the GBM, suggesting that heparanase may play an important role in HS reduction. The increased expression of heparanase in AN is in line with the findings in puromycin aminonucleoside nephrosis, passive Heymann nephritis and anti-GBM nephritis [15-18]. The loss of glomerular HS is also in accord with prior studies in proteinuric renal disease [7-9]. Our current data on a consistent association between HS and heparanase suggests that heparanase is an important factor involved in the breakdown of HS and thereby in the development of proteinuria. The pathogenetic potential for heparanase in proteinuria is supported by a recent study showing that inhibition of heparanase prevented both proteinuria and loss of HS in passive Heymann nephritis [16]. Moreover, heparin and heparin derivatives which inhibit heparanase, have been shown to exert antiproteinuric effects in diabetic nephropathy, further supporting the impact of heparanase [27].

Treatment with an ACE-inhibitor or AT1-antagonist ameliorates proteinuria in both human and experimental renal diseases and provides renoprotection [28-31]. Antiproteinuric treatment with AT1A in this study led to a partially restored glomerular HS expression. This effect of AT1A on HS expression in AN is comparable with the effect of ACE inhibition which preserved glomerular HS expression in rats with AN [9]. Along with the restored HS expression after treatment with AT1A, we observed a markedly reduced glomerular heparanase expression. In vitro experiments show that angiotensin II reduces HS expression in the extracellular matrix of human podocytes, raising the possibility of a direct effect [32].

We used three different approaches to study the expression of glomerular heparanase and HS in adriamycin nephropathy. The first was a unilateral model with a relatively low (1.5 mg/kg BW) adriamycin dosis, in order to allow good resolution over time. In the second study, the acute bilateral AN model (5 mg adriamycin/kg BW) with DMTU treatment was used with short-term follow-up. Finally, in the chronic bilateral AN model with AT1 intervention we used 2 mg adriamycin/kg BW. Despite variations in the induction and severity of the model, the observed correlation between HS and heparanase was consistently present.

The changes in glomerular heparanase expression in adriamycin model, and its reversibility by RAS-blockade observed here could theoretically be due to different factors, namely: (i) ROS, (ii) angiotensin II, or (iii) proteinuria. Although the generation of ROS can be induced by angiotensin II [33-35], ROS are also thought to be induced by adriamycin per se. The beneficial effects of DMTU treatment during the induction phase of AN support a role for ROS in the induction of changed charge-selective properties of the GBM. The effects of RAS blockade, showing reversibility of heparanase up-regulation in established nephropathy, support a role for angiotensin II in heparanase expression. As to proteinuria, the time course study with early changes in heparanase and HS expression, which were stable during long-term follow-up in spite of prolonged exposure to proteinuria, suggest that increased heparanase and decreased HS expression are causal to proteinuria rather than a consequence. Furthermore, in a model of protein-overload nephropathy {van Timmeren, 2006 13 /id}, we could not demonstrate a reduction in HS or an increase in heparanase expression (data not shown).

In conclusion, in addition to reactive oxygen species, heparanase plays an important role in the loss of HS in AN. Heparanase expression is increased early in the time course of AN and shows a clear association with the loss of HS in the GBM and proteinuria, suggesting that heparanase is an important mediator of loss of glomerular HS and development of proteinuria in AN. These results suggest that angiotensin II has a role in heparanase induction and that reduction of heparanase and the subsequent restoration of glomerular HS contribute to the beneficial effects of RAS-blockade.

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